Issued June 21, 1909.

U. S. DEPARTMENT OF AGRICULTURE,

BUREAU OF ANIMAL INDUSTRY.—BULLETIN 113.

A. D. MELVIN, CHIEF OF BUREAU.

FILTRATION EXPERIMENTS WITH BACILLUS CHOLERÆ SUIS.

BY

C. N. McBRYDE, M. D.,
Senior Bacteriologist, Biochemic Division.

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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., January 30, 1909.

Sir: I have the honor to transmit herewith, and to recommend for publication as a bulletin of this Bureau, the accompanying manuscript entitled "Filtration Experiments with *Bacillus choleræ suis*," by Dr. C. N. McBryde, of the Biochemic Division.

The subject-matter of the paper, while technical in its character, has a direct bearing on the hog cholera problem. In a previous publication issued by this Bureau (Bulletin 72, The Etiology of Hog Cholera) it was first shown that the so-called hog cholera bacillus, *B. choleræ suis*, could not be regarded as the definite and sole cause of hog cholera, since the disease could readily be produced by an ultra-microscopic virus in the blood of sick hogs, which virus had repeatedly passed through a filter that effectually prevented the passage of *B. choleræ suis*.

This discovery naturally caused widespread discussion among scientific investigators upon this subject throughout the world, some of whom have questioned the accuracy of the findings published in the bulletin referred to. The experiments described in the present paper have been carried out to meet these criticisms and have resulted in confirming the correctness of the work previously published.

Respectfully,

A. D. Melvin,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

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INTRODUCTORY.

In the early part of 1905 Dorset, Bolton, and the writer[a] published a paper on the etiology of hog cholera, in which it was first definitely shown that hog cholera is due to a filterable virus and not to *Bacillus choleræ suis* as had been previously supposed. The conclusions reached in that paper were based on an extensive series of filtration experiments in which the blood serum of hogs sick of hog cholera was filtered through Berkefeld and Chamberland filters in order to exclude *B. choleræ suis*. In these experiments a large number of nonimmune, healthy hogs were injected with the filtered serum, which was carefully tested in every instance prior to injection for *B. choleræ suis*. The filtrates were proven to be free from that organism by either incubating the entire filtrate or else taking out large portions for subcultures, and also by the injection of large portions into guinea pigs and rabbits, animals which are extremely susceptible to *B. choleræ suis*. These experiments with the filtered serum of hogs sick of hog cholera and proven to be free from *B. choleræ suis* showed that if such serum was injected subcutaneously into well hogs it caused typical hog cholera with great regularity, whereas the same serum injected into small laboratory animals which are naturally very susceptible to *B. choleræ suis* was entirely without effect. In many of these experiments rabbits were injected with as much as 20 c. c. of the filtrates without any subsequent ill effects, whereas hogs injected with like amounts of the filtrates died of typical hog cholera.

These results, coupled with others which developed in the course of this investigation, led to the following conclusions: (1) That in the disease known as hog cholera there is some other etiological factor present besides *B. choleræ suis;* (2) that this other factor is an ultra-visible virus sufficiently small to pass through the pores of Berkefeld and Chamberland filters; (3) that this ultra-visible virus is the true cause of hog cholera, and that *B. choleræ suis*, when present in the blood and tissues of animals sick of hog cholera, is probably a secondary invader and at most an accessory factor in the disease.

These conclusions with regard to the etiology of hog cholera, being decidedly revolutionary in character, attracted widespread attention

[a] Dorset, M., Bolton, B. M., and McBryde, C. N. The Etiology of Hog Cholera. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 72. Washington, 1905.

among scientific investigators in other countries, and it is gratifying to state that our results have been confirmed by many well-known workers in various parts of the world. Thus in Germany the same results were obtained by Ostertag and Stadie in the Hygienic Institute of the Veterinary High School at Berlin, and by Uhlenhuth and his coworkers, Hübener, Xylander, and Botz, in the laboratories of the Imperial Board of Health at Berlin. Similar results were obtained by Hutyra in Austria, by Stockman and McFadyean in England, by Theiler in South Africa, and within the past year by Carré, Leclainche, and Vallée in France. In our own country our results have been confirmed by McClintock, Boxmeyer, and Siffer. It is thus seen that our conclusions with regard to the etiology of hog cholera are not lacking in confirmatory proof.

Certain criticisms, however, have been made of our earlier work on the etiology of hog cholera, referred to above. In 1907 Lourens,[a] subdirector of the State Serum Institute at Rotterdam, published an article on the filterability of *Bacillus suipestifer* in which he makes the statement that the so-called hog-cholera bacillus, known as *B. choleræ suis* or *B. suipestifer*, is capable of passing through filters of the Chamberland and Berkefeld types, composed, respectively, of unglazed porcelain and infusorial earth. He claims that the ability of *B. choleræ suis* to pass through filters of the types mentioned is due to a property which this bacillus possesses of breaking up into granules sufficiently small to pass through the pores of the filter. He also asserts that *B. choleræ suis* is the cause of hog cholera and that none of the investigators who have conducted filtration experiments to show that hog cholera is due to a filterable virus have afforded sufficient and convincing proof that the filtrates which they employed did not contain *B. choleræ suis*.

While we had no doubts as to the accuracy of our earlier filtration work, nor any doubt whatever that hog cholera is due to a filterable virus, especially since our earlier work has been confirmed by such well-known investigators as those cited above, we nevertheless felt that such criticisms should not be allowed to pass unnoticed, and it was with a view to meeting these criticisms that the experiments described in the present paper were undertaken.

In connection with our earlier work on the etiology of hog cholera the writer carried out a series of filtration experiments with cultures of *B. choleræ suis*. This was done as a further test of the efficiency of the filters used—that is, to determine whether filters of the Berkefeld and Chamberland types could be relied upon absolutely to restrain or keep back *B. choleræ suis*.

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[a] Lourens, Louis F. D. E. Untersuchungen über die Filtrierbarkeit der Schweinepest-bacillen (*Bac. suipestifer*). Centralblatt für Bakteriologie, abt. 1, orig., band 44, heft 5, pp. 420–427, Aug. 20; heft 6, pp. 504–512, Aug. 31; heft 7, pp. 630–648, Sept. 17. Jena, 1907.

Since the publication of Lourens's paper a second series of filtration experiments with bouillon cultures of *B. choleræ suis* has been carried out by the writer, and the results obtained, together with those obtained in the earlier experiments referred to above, none of which have hitherto been published, are now recorded in detail.

FILTERS USED.

The filters used were Berkefeld laboratory cylinders Nos. 5 and 7, and Pasteur-Chamberland bougies "F" and "B," and were of the same types as those used in the filtration experiments described in Bulletin 72.

A circular which accompanied the Pasteur-Chamberland bougies stated that these filters were manufactured in France and imported into this country, and this was also stated on the cardboard boxes in which the bougies were packed. The individual bougies were labeled in French as follows: "F [or "B"] Filtre Chamberland, Système Pasteur, H. B. & Cie., Choisy-le-Roi."

New filters were used for each experiment, except in the case of experiments 7 and 8 with the Pasteur-Chamberland filters, where filters were employed which had been used once and then cleansed according to the methods adopted by Lourens.

Before being used the filters were first tested by submerging them in water and then forcing compressed air through their walls. If the air came through the walls of the filter in the form of small bubbles or beads the filter was regarded as being free from flaws, but if large air bubbles formed on the sides this was taken as an indication of possible defects or flaws, and such filters were discarded. This method of testing the filters is similar to that employed by Lourens.

ARRANGEMENT OF FILTERS.

In the first three experiments with Berkefeld filters and the first three experiments with the Pasteur-Chamberland type the filters were attached to ordinary side-arm or suction flasks and the filtrates collected in one portion. In the remainder of the experiments the fractional method of filtration was used, for which a special form of apparatus was devised. The apparatus, which is shown in figure 1, may be used with either the Berkefeld or the Chamberland type of filter. It consists essentially of a double side-arm suction flask with which the filters are connected above by means of rubber stoppers and a protected outlet below connected with the lower end of the suction flask by a short piece of heavy black rubber tubing. The outlet consists of a glass tube surrounded by a glass shield which is open at the lower end and is plugged with cotton to protect the outlet tube from dust. The outlet is controlled by a pinchcock above.

One arm of the suction flask is connected by means of heavy rubber tubing with a vacuum gauge and suction pump. The other arm is connected by means of rubber tubing with a glass inlet tube plugged with cotton, the admission of air into the suction flask being controlled by means of a screw pinchcock. (See fig. 1.)

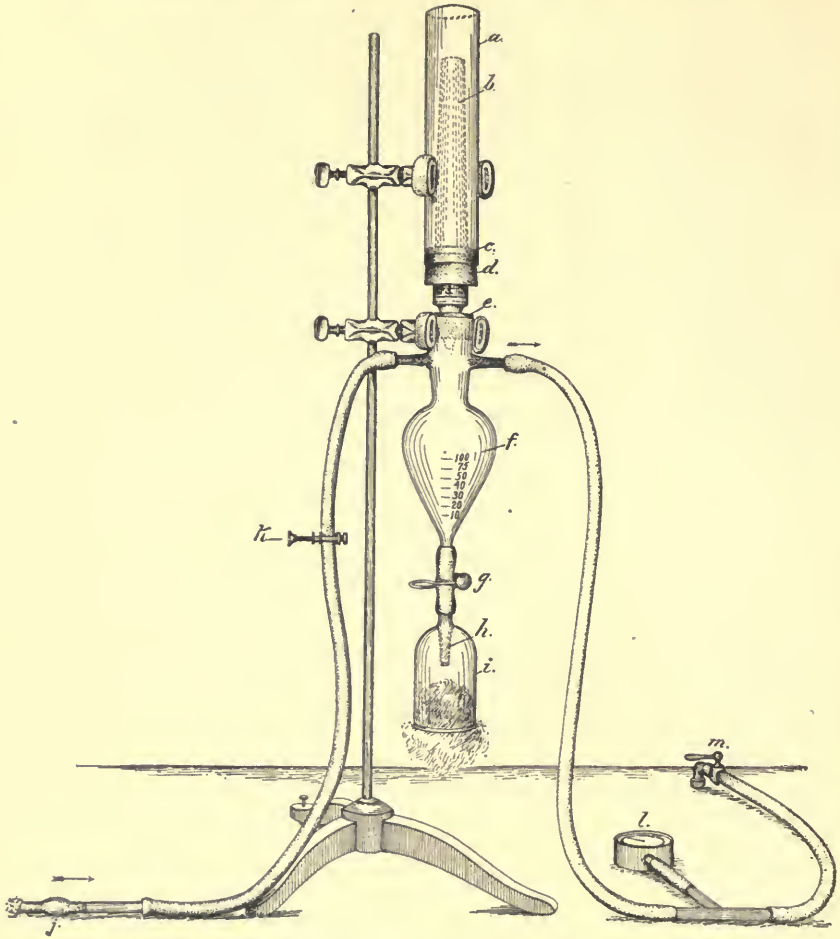

FIG. 1.—Apparatus for fractional filtration, designed for use with Pasteur-Chamberland or Berkefeld filters. *a*, Glass mantle surrounding filter; *b*, Chamberland filter; *c*, paraffin joint; *d* and *e*, rubber stoppers; *f*, double side-arm suction flask; *g*, pinchcock controlling outlet from suction flask; *h*, outlet tube surrounded by glass shield and attached to lower end of suction flask by means of short rubber tubing; *i*, glass shield fused to and surrounding outlet tube as a protection against contamination when the filtrates are drawn off; *j*, glass inlet tube plugged with cotton, for admitting air into suction flask; *k*, pinchcock governing the admission of air into flask; *l*, vacuum gauge; *m*, stopcock connected with vacuum pump.

TECHNIQUE EMPLOYED IN FILTRATIONS.

In conducting a filtration with the apparatus described, the pinchcocks *g* and *k* are first closed off and the vacuum applied at *m*. The filtration is then allowed to proceed until the filtrate reaches the

desired level in the suction flask *f*. As soon as the suction flask is exhausted of air the rubber tubing above the pinchcock *g* will be seen to collapse and will remain so as long as the vacuum is applied. When the filtrate reaches the desired level in the suction flask the vacuum is closed off and the air gradually admitted into the suction flask by means of the pinchcock *k* through the glass inlet tube plugged with cotton (*j*). The admission of air into the suction flask may be regulated by watching the rubber tubing above the pinchcock *g*, which expands as soon as the vacuum within the suction flask has been neutralized. The filtrate which has collected in the suction flask is now drawn off into a sterile Erlenmeyer flask by means of the pinchcock *g* through the glass outlet tube *h*. In withdrawing the filtrate an assistant removes the cotton plug from the glass shield surrounding the outlet tube, steadying the shield at the same time. The neck of the Erlenmeyer flask, which is to receive the filtrate, is then introduced within the shield beneath the outlet tube and the filtrate allowed to flow out by releasing the pinchcock above. The inlet tube is now closed off again by means of the pinchcock *k*, the vacuum reapplied at *m*, and another portion of the filtrate collected and drawn off as before. In this way as many fractions may be collected as are desired.

Dry heat is used for the sterilization of all glass portions of the apparatus except the glass mantle *a* surrounding the filter, which being outside of the filter does not require sterilization. Both arms of the suction flask should be plugged with cotton to prevent dust particles from being drawn into the flask. The filters and all rubber connections are sterilized by boiling in distilled water for from one-half hour to one hour. After the apparatus is set up all joints should be carefully paraffined.

CULTURES USED.

Five different strains of *Bacillus choleræ suis* were used. These cultures were obtained from different sources, either directly from hogs which died of hog cholera or from guinea pigs inoculated with cultures obtained from hogs which had died of hog cholera. The cultures were grown on the various laboratory media and their biological and morphological characters carefully noted. All of the cultures used were virulent for guinea pigs and rabbits.

A brief record of the different cultures is appended.

1. *Culture Gp 3998 S.*—This culture was obtained from guinea pig 3998, which died from the inoculation of a culture obtained from hog 1086. Hog 1086 was a nonimmune animal which served as a check in the Iowa experiments. The animal was exposed, together with certain other treated hogs, to a natural outbreak of hog cholera. As a result of the exposure the animal contracted the disease and died with typical cholera lesions, *B. choleræ suis* being obtained in pure

culture from the heart blood and spleen. A subculture obtained from agar plates made from the spleen of hog 1086 was used for the inoculation of guinea pig 3998. The guinea pig died within six days, and *B. choleræ suis* was recovered in pure culture from the spleen and heart blood. A subculture from agar plates made from the spleen of guinea pig 3998 was cultivated on the various laboratory media and found to correspond with *B. choleræ suis* in all respects. This culture, which we have designated Gp 3998 S, was used in the first four experiments with Berkefeld filters, and the first two experiments with Pasteur-Chamberland filters.

2. *Culture Gp 4692 S.*—Obtained from the spleen of guinea pig 4692, which died from the injection of 1 c. c. of unfiltered, undiluted blood serum of hog 1208, an experiment hog carrying the Scribner strain of disease in the experiments at Washington (see "Experiments with tail blood," Bulletin 72, page 38). This culture was used in a number of the culture experiments described in Bulletin 72 and was found to correspond with *B. choleræ suis* in all of its cultural characters.

3. *Culture II 2199 S.*—This culture was obtained directly from hog 2199, which served as a check in the Iowa immunity experiments. The animal died from the injection of 2 c. c. of unfiltered disease-producing blood from the Scribner and Syphax strains of disease. A description of the Scribner and Syphax outbreaks may be found in Bulletin 102[a] of this Bureau. The autopsy on hog 2199 revealed typical cholera lesions and *B. choleræ suis* was obtained in pure culture from the spleen. This culture, designated as II 2199 S, was cultivated on the different laboratory media and was found to correspond in all of its cultural characters with *B. choleræ suis.*

4. *Culture II 2450 S.*—This was obtained directly from hog 2450, a western hog used in the Iowa experiments. Hog 2450 was an uninoculated check and was placed in the exposure pen (for description of exposure pen see Bulletin 102, page 12) along with certain treated hogs in order to test the virulence of the disease prevailing in the exposure pen at the time. The animal contracted hog cholera in the usual time and died with characteristic lesions of the disease. Cultures from the spleen were grown on the different laboratory media and found to correspond in all respects with *B. choleræ suis.*

5. *Culture II 2228 S.*—This culture was obtained directly from hog 2228, which died as a result of an injection of 5 c. c. of defibrinated blood obtained from an outbreak of hog cholera in Iowa. Hog 2228 exhibited characteristic cholera lesions at autopsy and agar plates from the spleen revealed *B. choleræ suis.* This culture, like all of the others, was cultivated on the various laboratory media and was found to correspond with *B. choleræ suis* in all of its cultural characters.

[a] Dorset, M., McBryde, C. N., and Niles, W. B. Further Experiments Concerning the Production of Immunity from Hog Cholera. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 102. Washington, 1907.

EXPERIMENTS WITH BERKEFELD FILTERS.

EXPERIMENT 1.

A flask containing 400 c. c. of neutral beef broth was inoculated with culture Gp 3998 S and placed in the incubator. At the end of twenty-four hours the flask was well clouded and 200 c. c. of the culture was then passed through a Berkefeld laboratory cylinder No. 5. The vacuum used was 20 inches and the time occupied in filtration was one and one-half hours. The filtrate was collected in one portion in an ordinary side-arm flask and placed at once in the incubator. At the end of eighteen hours the filtrate, which came through the filter perfectly clear, was distinctly clouded. Agar plates from the filtrate showed *B. choleræ suis* in pure culture. A subculture made from the agar plates was grown on the different laboratory media and found to correspond in all cultural characteristics with the original culture. The results obtained in this experiment show that *B. choleræ suis* passed through the Berkefeld filter in the first 200 c. c. of filtrate.

EXPERIMENT 2.

A flask of neutral beef broth was inoculated with culture Gp 3998 S and incubated at 37° C. for twenty-four hours. The culture showed well-marked clouding and a microscopic examination showed the organisms to be actively motile. Filtration was made through a Berkefeld laboratory cylinder No. 7. The amount of culture filtered was 100 c. c. and the time occupied in filtration was one and one-half hours. The filtration was conducted under a vacuum of 22 inches. The filtrate was collected in one portion and was immediately placed in the incubator. After twenty-four hours in the incubator the filtrate showed distinct clouding and agar plates revealed a pure culture of *B. choleræ suis*. A subculture made from the agar plates was cultivated on the different laboratory media. This culture was found to correspond with the original culture in all of its cultural characteristics. In this experiment *B. choleræ suis* passed through the Berkefeld filter in the first 100 c. c. of filtrate.

EXPERIMENT 3.

This experiment was practically a repetition of experiment 2 and the results obtained were the same. The filter used was a Berkefeld laboratory cylinder No. 7. The amount of culture filtered was 100 c. c., which required one and one-half hours to pass through the filter. The vacuum employed was 23½ inches. The entire filtrate was collected in one portion, as in the two preceding experiments. After forty-eight hours in the incubator the filtrate showed heavy clouding and agar plates revealed a pure culture of *B. choleræ suis*. A subculture was made from the agar plates and grown on the different laboratory media. This culture was found to correspond in all cultural characteristics with the original culture.

EXPERIMENT 4.

In this experiment the fractional method of filtration was used, the filtrates being collected in large sterile test tubes. In carrying out the experiment a twenty-four hour culture of Gp 3998 S, grown on neutral beef broth, was used. Filtration was made through a Berkefeld laboratory cylinder No. 7. The amount of culture filtered was 120 c. c. and the actual time consumed in the filtration was one hour and eighteen minutes. The suction varied from 20½ to 21½ inches. The details of the experiment are shown in the following table:

Table showing details of experiment 4.

Portion.	Amount.	Time consumed in passing filter.	Suction.	Result of incubation.
	c. c.	*Minutes.*	*Inches.*	
I	20	4	20½	Remained clear.
II	20	7	20½	Do.
III	20	10	20½	Do.
IV	20	12	21	Do.
V	10	7	21	Do.
VI	10	10	21	Do.
VII	10	11	21½	Clouded.
VIII	10	17	21½	Do.

The test tubes containing the filtrates were placed in the incubator immediately after filtration. At the end of twenty-four hours portions VII and VIII showed very slight clouding; other tubes remained clear. At the end of forty-eight hours portions VII and VIII showed increased clouding, while the others remained clear. Agar plates were made from portions VII and VIII and revealed pure cultures of *B. choleræ suis*. Subcultures from the agar plates were grown on the various laboratory media and found to correspond with the original culture. Portions I to VI were kept in the incubator for two weeks and remained perfectly clear. Hanging-drop preparations and agar plates from these portions failed to reveal any organisms.

In this experiment the Berkefeld cylinder held back the organisms until 100 c. c. of the culture had been filtered, but the first portion of 10 c. c. collected after 100 c. c. of culture had passed the filter showed *B. choleræ suis*.

EXPERIMENT 5.

In this experiment a twenty-six hour bouillon culture (II 2450 S) was used. The reaction of the beef broth in this instance was 1 per cent acid. The culture showed well-marked, uniform clouding, and a microscopic examination previous to filtration revealed active motility. Filtration was made through a Berkefeld cylinder No. 5 arranged for fractional filtration. The vacuum employed varied from 20 to 23 inches. It was the intention to filter several hundred cubic centimeters of the culture and collect the filtrate in separate

fractions, as in the preceding experiment, but the filtration proceeded very slowly and was discontinued at the end of forty-five minutes after 50 c. c. of culture had passed the filter. The filtrate was drawn off in a small sterile Erlenmeyer flask and placed in the incubator. At the end of eighteen hours the filtrate showed a distinct, uniform clouding and agar plates revealed a pure culture of *B. choleræ suis*.

In this experiment *B. choleræ suis* passed through a Berkefeld cylinder in the first 50 c. c. of filtrate.

SUMMARY OF EXPERIMENTS WITH BERKEFELD FILTERS.

The results of the experiments with Berkefeld filters are summarized in the following table:

Summary of Berkefeld filtrations.

No. of experiment.	Date begun.	Grade of filter.	Culture used.			Amount filtered.	Time occupied in filtration.	Vacuum.	Result.
			No.	Age.	Medium.				
	1904.			*Hrs.*		*c. c.*	*h. m.*	*Inches.*	
1.....	Feb. 28	No. 5..	Gp 3998 S...	24	Neutral beef broth.	200	1 30	20	*B. choleræ suis* in filtrate.
2.....	Mar. 7	No. 7..	.....do.....	24	.....do.....	100	1 30	22	Do.
3.....	Mar. 9	No. 7..	.....do.....	24	.....do.....	100	1 30	23½	Do.
4.....	Mar. 15	No. 7..	.....do.....	24	.....do.....	120	1 18	20½–21½	*B. choleræ suis* in filtrate after 100 c. c. had passed filter.
	1908.								
5.....	June 12	No. 5..	II 2450 S.....	26	+ 1 beef broth..	50	45	20 –23	*B. choleræ suis* in filtrate.

DISCUSSION OF BERKEFELD FILTRATIONS.

In the foregoing experiments five Berkefeld cylinders, all of them new, were tested with bouillon cultures of *B. choleræ suis*, and in each instance the organism passed through the walls of the filter. In the case of one cylinder (experiment 4) the organism did not appear in the filter until 100 c. c. of the culture had been filtered. In one instance (experiment 5) the organism appeared in the first 50 c. c. of filtrate. With two of the cylinders (experiments 2 and 3) the organism passed through the walls of the filter in the first 100 c. c. of filtrate, and in the case of the remaining filter (experiment 1) the organism appeared in the first 200 c. c. of filtrate.

These results would indicate that in the case of liquid cultures or relatively heavy suspensions of small micro-organisms like *B. choleræ suis* the small laboratory Berkefeld filter can not be relied upon to keep back the organisms when any considerable amount of the material is filtered.

That the results obtained in these experiments conform with those obtained by other investigators who have tested the Berkefeld filter is shown by the following brief review of the literature relating to Berkefeld filters.

In 1902 Wherry[a] found that the bacillus of pneumonia in guinea pigs, an organism somewhat shorter than, but very nearly as thick as, *B. choleræ suis*, would pass through the pores of a Berkefeld cylinder No. 5. Bouillon cultures one and three days old were used in these experiments, and the organism appeared in the filtrate after from 75 c. c. to 80 c. c. of the culture had been filtered.

Pfuhl[b] in 1903 made a series of tests with four small Berkefeld cylinders and found that of these only one could be depended on with safety to deliver the first 100 c. c. of filtrate germ free. One of the filters gave 50 c. c. of germ-free filtrate, but no more; the other two filters failed to give even 50 c. c. of germ-free filtrate. The cultures used in these tests were *B. coli communis* and a vibrio of the same size as the cholera spirillum.

In a parallel series of experiments with large Berkefeld cylinders, which have thicker walls, Pfuhl found that 50 per cent of these filters were unable to restrain organisms approximating the size of *B. typhosus* and *B. dysenteriæ*.

Novy and MacNeal[c] in 1904 found that *Trypanosoma lewisi* could be passed through a Berkefeld filter.

Novy and Knapp[d] in 1906 showed that even so large an organism as *Spirochæta obermeieri*, which is 7 to 19 microns or more in length, will pass through the small Berkefeld filters under a pressure of 50 pounds.

Bulloch, Craw, and Atkin[e] in 1908 tested ten Berkefeld filters with tap water and found that only one gave a sterile filtrate on the first day under a maximum pressure of 32.5 pounds to the square inch. The remaining nine yielded contaminated filtrates within fifteen minutes, or practically as soon as the filters were started.

EXPERIMENTS WITH PASTEUR-CHAMBERLAND FILTERS.

EXPERIMENT 1.

A flask of neutral beef broth was inoculated with culture Gp 3998 S and incubated for twenty-four hours. At the end of this time the culture showed well-marked clouding and a microscopic

[a] Wherry, William B. Experiments on the permeability of the Berkefeld filter and the Pasteur-Chamberland bougie to bacteria of small size. Journal of Medical Research, vol. 8 (n. s., vol. 3), No. 2, pp. 322–328. Boston, Nov., 1902.

[b] Pfuhl, E. Ergebnisse einer erneuten prüfung einiger kieselgur- und porzellanfilter auf keimdichtigkeit. Festchrift zum 60th Geburtstag von Robert Koch, pp. 75–86. Jena, 1903.

[c] Novy, F. G., and MacNeal, Ward J. On the filtration of trypanosomes. Michigan Academy of Science, Sixth Report, p. 180. Lansing, 1904.

[d] Novy, F. G., and Knapp, R. E. Studies on *Spirillum obermeieri* and related organisms. Journal of Infectious Diseases, vol. 3, No. 3, pp. 291–393. Chicago, May 18, 1906.

[e] Bulloch, William, Craw, J. Anderson, and Atkin, E. E. On the relative efficacy of the Doulton, Berkefeld, and Brownlow filters. Journal of Hygiene, vol. 8, No. 1, pp. 63–69. Cambridge, Jan., 1908.

examination revealed active motility. The culture was then passed through an F bougie arranged to deliver into an ordinary side-arm suction flask. The amount of culture filtered was 200 c. c., the vacuum employed was 23 inches, and the time consumed in passing through the filter was thirty-five minutes. The entire filtrate was collected in one portion. When the filtration was complete the side-arm flask containing the filtrate was disconnected from the filter, plugged with a sterile cotton plug, and immediately placed in the incubator. At the end of eight days the filtrate was found to be perfectly clear, and a careful microscopic examination at this time failed to reveal any micro-organisms. A small portion of the filtrate was removed at this time by means of a sterile pipette and the remainder replaced in the incubator. A guinea pig was injected subcutaneously with 0.5 c. c. of the portion removed and showed no ill effects from the inoculation. The filtrate was kept in the incubator for six weeks and remained perfectly clear. The filtrate was again subjected to a careful microscopic examination before being discarded, and was apparently perfectly sterile.

EXPERIMENT 2.

A flask containing 400 c. c. of neutral beef broth was inoculated with culture Gp 3998 S and placed in the incubator for twenty-four hours. One-half of the culture (200 c. c.) was then passed through an F bougie and the other half through a B bougie, the two bougies being fitted to ordinary side-arm suction flasks. A vacuum of 21 to 22 inches was used, and the time required for the two filtrations, which were conducted simultaneously, was approximately forty-five minutes. The two side-arm flasks containing the filtrates were immediately placed in the incubator, where they remained for six weeks without showing any signs of bacterial growth. Before being finally discarded both filtrates were subjected to a careful microscopic examination, but no micro-organisms could be demonstrated in either hanging-drop or stained preparations.

EXPERIMENT 3.

A seven-day bouillon culture (Gp 4692 S) was used in this experiment. Filtration was made through a Pasteur-Chamberland B bougie. The filtration was discontinued after 100 c. c. of filtrate had passed the filter. The vacuum gauge recorded 20 inches during the filtration, which occupied thirty-seven minutes. The filtrate was collected in one portion and immediately placed in the incubator. After two weeks in the incubator the filtrate showed a very slight opalescence, but a careful microscopic examination failed to reveal any micro-organisms and the opalescence was attributed to a slight precipitate from the beef broth. In order to test further the ster-

ility of the filtrate, rabbits were injected as follows: Rabbit 1571 received 1 c. c. of filtrate intravenously; rabbit 1332, 3 c. c. intravenously; rabbit 1570, 5 c. c. intravenously; rabbit 1574, 5 c. c. intraperitoneally. These animals were kept under observation for several months and showed no ill effects whatever from the injections of filtrate.

The possibility now suggested itself that *Bacillus choleræ suis* might pass through the walls of the Pasteur-Chamberland filter in some form which would not develop in vitro, but which might develop within the animal body. In order to test this point collodion sacs were prepared according to the methods of Grubbs and Francis,[a] of the United States Public Health and Marine-Hospital Service, and McCrae,[b] of the Johns Hopkins Hospital. Sacs prepared according to each of these methods were filled with the filtrate and inserted into the abdominal cavities of rabbits. After an interval of several weeks the animals were killed and the sacs removed. The filtrate within the sacs showed no apparent clouding, and a microscopic examination of the contents of the sacs and cultures from the same failed to reveal any micro-organisms.

EXPERIMENT 4.

A forty-eight-hour bouillon culture (H 2199 S) was used in this experiment. Filtration was made through a Pasteur-Chamberland F bougie. In this experiment and in those which follow the fractional method of filtration was used, the filtrates being collected in separate portions in small sterile Erlenmeyer flasks, which were placed in the incubator as soon as the filtrations were completed.

Table showing details of experiment 4.

Portion	Amount.	Time occupied in passing filter.	Vacuum.	Result of incubation.
	c. c.		*Inches.*	
I	75	5 minutes	21	Clouding at 48 hours.
II	75	8 minutes	21	Do.
III	75	10 minutes	21	Clouding at 7 days.
IV	75	35 minutes	21	Clouding at 48 hours.
V	35	3 hours	21	Remained clear.
VI	10	Over night	No suction.	Do.

During the filtration of portions I, II, and III the bougie was entirely covered; during the filtration of portions IV, V, and VI it was only partly covered. Only about one-third of the filter was

[a] Grubbs, S. B., and Francis, Edward. Laboratory technique. Collodion sacs. U. S. Treasury Department, Public Health and Marine-Hospital Service, Hygienic Laboratory, Bulletin 7. Washington, 1902.

[b] McCrae, John. Notes upon the agglutinations obtained by intraperitoneal insertion of celloidin capsules containing bacilli and upon a mode of preparing such capsules. Journal of Experimental Medicine, vol. 5, No. 6, pp. 635-642. New York, Oct. 1, 1901.

covered with culture during the filtration of portion V, and this, together with the fact that the pores of the filter had become clogged, explains the length of time required for this portion to pass the filter.

After drawing off portion V the suction was disconnected and the apparatus left in place overnight. On the following morning it was found that approximately 10 c. c. of the culture had passed the filter, and this portion was drawn off and incubated along with the other fractions.

Portions I, II, and IV showed clouding at forty-eight hours. Microscopic examinations and agar plates showed in each case pure cultures of a micrococcus corresponding in morphology to *Staphylococcus pyogenes albus*, and probably due to outside contamination at the time these portions were drawn off. Guinea pigs weighing approximately 350 grams each were injected with 2 c. c. each of these portions and exhibited no ill effects from the injections.

Portion III became cloudy after one week in the incubator. Microscopic examination and agar plates revealed a pure culture of a micrococcus similar to that found in portions I, II, and IV. The injection of 2 c. c. of this portion into a guinea pig was without effect.

Portion V was kept in the incubator for ten days and remained perfectly clear. A portion of this filtrate was removed at the end of ten days by means of a sterile pipette, and a rabbit weighing 2,380 grams was given a subcutaneous injection of 10 c. c. without any subsequent ill effects. After drawing off the portion for the injection of the rabbit the remainder of the filtrate was inoculated with culture H 2199 S in order to see whether the filtrate would still furnish a suitable medium for the growth of *B. choleræ suis*. The inoculated portion was replaced in the incubator and showed well-marked clouding at twenty-four hours.

Portion VI was kept in the incubator for ten days and remained perfectly clear. It was then inoculated with culture H 2199 S, and showed well-marked clouding at twenty-four hours, thus demonstrating that *B. choleræ suis* would have grown in this filtrate had it passed through the pores of the filter.

The clouding of portions I, II, III, and IV in this experiment was due either to imperfect sterilization of the apparatus or to outside contamination at the time these portions were drawn off. That the clouding of these portions was not due to the passage of *B. choleræ suis* through the filter is shown by the fact that guinea pigs inoculated with these portions showed no ill effects from the inoculations.

It should be stated in this connection that the experiments described in this paper were conducted in a large general bacteriological workroom, where the conditions were more or less favorable for the outside contamination of the filtrates, owing to the constant passing of other workers and the impossibility of avoiding drafts and air currents.

EXPERIMENT 5.

In this experiment an eighteen-hour bouillon culture (H 2199 S) was used. The reaction of the bouillon in this experiment and those which follow was 1 per cent acid. Filtration was made through a Pasteur-Chamberland F bougie. The filtrate was collected in separate portions in small sterile Erlenmeyer flasks, which were placed in the incubator as soon as the filtration was completed. The bougie was kept entirely covered with culture during the filtration. The amount of culture filtered was 300 c. c.

Table showing details of experiment 5.

Portion	Amount.	Time occupied in filtration.	Vacuum.	Result of incubation.
	c. c.	*Minutes.*	*Inches.*	
I	75	2	20½	Clouded.
II	75	2	20½	Remained clear.
III	75	3	20½	Do.
IV	75	4	20½	Do.

Portion I showed clouding after forty-eight hours in the incubator. Microscopic examination and agar plates revealed a pure culture of a large spore-bearing organism resembling *Bacillus subtilis*. A guinea pig weighing 350 grams was injected with 2 c. c. of this portion but showed no ill effects from the injection. The organism noted in this flask was evidently due to outside contamination at the time the portion was drawn off.

Portions II, III, and IV were kept in the incubator for two weeks and remained perfectly clear. Two portions of 10 c. c. each were then removed from III and IV by means of sterile pipettes and injected into rabbits, as follows: Rabbit 2224 (weight 2,620 grams) received 10 c. c. of portion III; rabbit 2223 (weight 1,801 grams) received 10 c. c. of portion IV. The animals were kept under observation for several months, and showed no ill effects whatever from the injections.

After withdrawing portions from III and IV for the injection of rabbits, the remaining portions of these filtrates were inoculated with *B. choleræ suis* (culture H 2199 S) and returned to the incubator. After twenty-four hours both flasks showed well-marked clouding, and hanging-drop preparations and agar plates showed pure cultures of *B. choleræ suis*, proving that the absence of growth in these portions after filtration was not due to exhaustion of the bouillon.

EXPERIMENT 6.

Two flasks, each containing 400 c. c. of beef broth, were inoculated with *B. choleræ suis* (H 2199 S) and incubated for twenty-four hours. The contents of the two flasks were then poured together into a

large balloon flask and well mixed. The object of this was to give a sufficient quantity of culture to keep the bougie covered during the entire filtration. Filtration was made through a Pasteur-Chamberland F bougie, which was kept entirely covered by the culture throughout the filtration. The filtrate was collected in separate portions of 75 c. c. each in seven flasks, the total amount of culture filtered being 525 c. c.

Table showing details of experiment 6.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.	*Minutes.*	*Inches.*	
I	75	2	20	Clouded.
II	75	3	20	Remained clear.
III	75	7	23–24	Do.
IV	75	9	23½–24	Do.
V	75	16	24–25	Do
VI	75	19	25	Do.
VII	75	27	25	Do.

Portion I showed slight clouding at forty-eight hours, and a microscopic examination revealed a rather long, slender, nonmotile bacillus, apparently in pure culture. The flask was returned to the incubator and allowed to remain for six days, at the end of which time it showed well-marked, uniform clouding. Hanging-drop preparations and agar plates revealed the same bacillus that had been noted at forty-eight hours. A guinea pig, weighing approximately 350 grams, was injected with 2 c. c. of this portion after incubation for six days, but showed no ill effects as a result of the injection. The clouding of this portion was thus proven to be due to a contaminating organism and not to *B. choleræ suis.*

Portions II, III, IV, V, VI, and VII were incubated for twelve days, and remained perfectly clear. Rabbits were injected with portions VI and VII as follows: Rabbit 2222 (weight 2,500 grams) received 10 c. c. of portion VI; rabbit 2227 (weight 2,088 grams) received 10 c. c. of portion VII. These animals were kept under observation for several months, and remained perfectly well.

After removing portions for the injection of rabbits, the remaining portions of VI and VII were inoculated with *B. choleræ suis* (II 2199 S) and replaced in the incubator. Both flasks showed well-marked clouding at twenty-four hours, and microscopic preparations and agar plates revealed pure cultures of *B. choleræ suis.* This was done in order to prove that the bouillon had not been exhausted, but was still a suitable medium for the growth of *B. choleræ suis.*

EXPERIMENT 7.

In this experiment and in the one which follows Pasteur-Chamberland bougies were employed which had already been used in the preceding experiments. The bougies were cleansed according to

methods recommended by Lourens before being used the second time. Lourens, it should be stated, did not use new filters in all of his experiments, but adopted certain methods for cleansing his filters, and the two experiments which follow were designed with the view to determining whether the methods which he adopted for cleansing his filters could have had any effect on the permeability of the filters and so have influenced his results.

In carrying out experiment 7 an eighteen-hour bouillon culture of *B. choleræ suis* (H 2199 S) was used. The bougie used was a Pasteur-Chamberland F which had been used once before in experiment 2. Before being used the second time it was treated by the method which Lourens first used for cleansing his filters, as follows: The filter was first washed with cold tap water, boiled for ten minutes in 1 per cent hydrochloric acid, then boiled for ten minutes in a 3 per cent sodium carbonate solution, after which a liter or more of cold distilled water was passed through the filter until the filtrate showed only a faint alkaline reaction. The filter was sterilized by boiling for one-half hour in distilled water. The filtrate was collected in separate portions. The amount of culture filtered was 750 c. c.

Table showing details of experiment 7.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.		*Inches.*	
I	75	1½ minutes.	20½	Remained clear.
II	75	2 minutes.	20 –18	Do.
III	75	3 minutes.	18 –21	Do.
IV	75	6 minutes.	21 –22½	Clouded.
V	75	9 minutes.	22½	Remained clear.
VI	75	12 minutes.	22½–21½	Do.
VII	75	16 minutes.	21½–22	Do.
VIII	75	1½ hours....	22	Do.
IX	150	Overnight...	No suction.	Do.

During the filtration of the first seven portions the bougie was entirely covered with culture; during the filtration of portion VIII the bougie was partly exposed. After portion VIII was drawn off the vacuum tubing was disconnected from the side-arm flask and the apparatus left in place overnight. On the following morning, after standing for eighteen hours, 150 c. c. of filtrate had collected in the side-arm flask. This portion, which passed through the filter during the night, was then drawn off and placed in the incubator, together with the other portions.

Portions I, II, III, V, VI, VII, VIII, and IX remained perfectly clear after eleven days in the incubator. Rabbits were then injected subcutaneously with portions VI and VII, as follows: Rabbit 2225 (weight 2,288 grams) received 10 c. c. of portion VI; rabbit 2226 (weight 2,185 grams) received 10 c. c. of portion VII. These animals were kept under observation for several months, and showed no ill effects from the inoculations.

Careful microscopic examinations were made of several of these filtrates, both in hanging-drop and in smear preparations stained with dilute carbol-fuchsin, and small, coccus-like bodies were noted, but cultures from the same portions gave negative results.

Portion IV showed clouding after four days, and agar plates revealed a pure culture of a medium-sized micrococcus corresponding in morphology with *S. pyogenes albus*. A guinea pig was inoculated with 2 c. c. of this portion after clouding had developed, and showed no ill effects from the inoculation. The growth in this flask was evidently due to outside contamination when the portion was drawn off. This is shown by the fact that all of the portions drawn off before this one, as well as all of those drawn off after it, were sterile.

Several of the filtrates after being incubated for eleven days with negative results were inoculated with *B. choleræ suis* and gave well-marked clouding at twenty-four hours, showing that the culture medium was still suitable for the growth of *B. choleræ suis*.

EXPERIMENT 8.

The culture used in this experiment was a twenty-four-hour bouillon of *B. choleræ suis* (II 2199 S). Filtration was made through a Pasteur-Chamberland F bougie which had been used once in experiment 6. The filter was cleansed according to the method finally adopted by Lourens as yielding the most satisfactory results. Following this method, the outside of the filter was first washed with cold tap water; a liter of cold distilled water was then passed through the filter; next a liter of potassium permanganate solution (1 gm. $KMnO_4$, 6.5 gms. HCl, 1,000 c. c. water) was drawn through the filter; following the permanganate, a like amount of oxalic-acid solution (10 gms. oxalic acid to 1,000 c. c. water) was passed through the filter; hot water was next drawn through the filter until the filtrate was acid-free, and finally a liter of cold distilled water was passed through. Before being used the filter was sterilized in the usual manner by boiling for one-half hour in distilled water. The filtrate was collected in separate portions, the total amount of culture filtered being 525 c. c. During the filtration the entire filter was covered with the culture.

Table showing details of experiment 8.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.	*Minutes.*	*Inches.*	
I	75	2½	23½	Remained clear.
II	75	6	23½	Do.
III	75	9	23½	Do.
IV	75	13	23½	Do.
V	75	22	23½–17	Do.
VI	75	30	8	Do.
VII	75	40	8–10	Do.

Portions I to VII, inclusive, remained perfectly clear after ten days in the incubator, at the end of which time two rabbits were injected subcutaneously with portions VI and VII, as follows: Rabbit 2118 (weight 1,875 grams) received 10 c. c. of portion VI; rabbit 2119 (weight 1,892 grams) received 10 c. c. of portion VII. The animals were kept under observation for several months, but showed no ill effects from the injections. After withdrawing sufficient quantities of portions VI and VII for the injection of the rabbits, the remainders of these portions were inoculated with *B. choleræ suis* and showed well-marked clouding after twenty-four hours.

Portions I, II, III, IV, and V were left in the incubator for six weeks and remained perfectly clear. A microscopic examination of several of these portions revealed small bodies like those noted in experiment 7, but cultures from these portions yielded negative results.

EXPERIMENT 9.

A twenty-six hour bouillon culture of *B. choleræ suis* (H 2450 S) was used in this experiment. The filter used was a new Pasteur-Chamberland F bougie. The filtrate was collected in seven portions of 50 c. c. each, the total amount of culture filtered being 350 c. c. The bougie was kept covered with culture during the entire filtration.

Table showing details of experiment 9.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.		*Inches.*	
I	50	40 seconds	23	Remained clear.
II	50	1½ minutes	23	Do.
III	50	2 minutes	23	Do.
IV	50	4½ minutes	23	Do.
V	50	6 minutes	20	Do.
VI	50	7½ minutes	20	Do.
VII	50	17 minutes	20	Do.

The filtrates were placed in the incubator immediately after filtration and showed no perceptible clouding after prolonged incubation.

Portions I, II, VI, and VII were left in the incubator for six weeks and remained perfectly clear. Cultures were made from these portions on various laboratory media, including Martin's serum-broth, with negative results.

Portion III was removed from the incubator at the end of two weeks and was found to be perfectly clear. This portion was subjected to a careful microscopic examination. In hanging-drop preparations small bodies having somewhat the appearance of micrococci were noticed. In preparations stained with dilute carbol-fuchsin which had been carefully filtered through double filters to remove all dirt and granules, small bodies were also noted; these bodies varied in size from minute points to bodies 0.6 μ in diameter. No bacilli were noted in any of the preparations.

Cultures were made from this portion as follows: Two tubes of neutral agar, two tubes of neutral beef broth, and two tubes of Martin's bouillon with normal hog serum added were inoculated with 1 c. c. each, but none of these cultures showed any growth after two weeks' incubation. Martin's bouillon, with the addition of serum, was the medium upon which Nocard and Roux[a] in 1898 first succeeded in cultivating outside of the animal body the organism of bovine pleuro-pneumonia, an organism which up to that time had resisted all attempts at cultivation and had been classed among the ultravisible viruses. Nocard and Roux employed Martin's bouillon with the addition of beef or rabbit serum, whereas in the present experiments hog serum was used as affording a more suitable medium for the possible growth of any living particles or bodies which might have passed through the pores of the filters.

Two hogs weighing from 30 to 40 pounds each were injected with portion III as follows: Hog 2351 received an intravenous injection in ear vein of 10 c. c. of portion III; hog 2352 received a subcutaneous injection in groin of 20 c. c. of portion III. These animals were kept under observation for several months and remained perfectly well.

In explaining his failure to produce hog cholera by the subcutaneous injection of cultures of *B. choleræ suis* and the high degree of virulence which the blood of animals sick of hog cholera exhibits when injected subcutaneously, Theobald Smith[b] advanced the theory that the blood coagulates in the connective tissues and serves as a food for the bacilli which it incloses and at the same time protects them against the action of the leucocytes. With this theory in mind, and in order to determine whether the coccus-like bodies noted in this filtrate possessed any significance—that is, whether they were capable of further development in the animal body—the following experiment was carried out: Ten cubic centimeters of blood was drawn from the tail of a healthy hog under aseptic conditions and collected in a large sterile test tube, the interior of which had received a coating of sterile olive oil to prevent coagulation. To this blood was immediately added 10 c. c. of portion III. The mixture of blood and filtrate was then drawn up into a sterile syringe and at once injected into the groin of a healthy hog (No. 2354) before coagulation had time to take place. Now, if the coccus-like bodies noted in this filtrate had been capable of developing into bacillary forms, as Lourens claimed for those which he observed, they were certainly afforded in this experiment, according to Smith's theory, a good opportunity to develop within the animal body. This hog, however, was kept under observation for several months and remained perfectly well.

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[a] Nocard, E. I. E., and Roux. Le microbe de la péripneumonie. Annales de l'Institut Pasteur, tome 12, No. 4, pp. 240–262, Paris, Apr. 25, 1908.

[b] Hog Cholera: Its History, Nature, and Treatment. United States Department of Agriculture, 1889.

Portion IV was removed from the incubator at the end of three weeks. Hanging-drop and smear preparations stained with dilute carbol-fuchsin showed bodies similar to those noted in portion III, but no bacilli. Two rabbits, weighing approximately 2,000 grams each, were injected as follows: Rabbit 2306 received an intravenous injection of 3 c. c. of portion IV; rabbit 2307 received a subcutaneous injection of 10 c. c. of portion IV. Both animals remained well and showed no ill effects as a result of the injections.

Portion V was removed from the incubator at the end of four weeks. Microscopic examination revealed the same bodies noted in portions III and IV, but there had been no apparent multiplication of these bodies and no bacilli were observed. Two rabbits, weighing approximately 2,300 grams each, were injected as follows: Rabbit 2301 received an intravenous injection of 3 c. c. of portion V; rabbit 2308 received a subcutaneous injection of 10 c. c. of portion V. Both animals remained well, showing no ill effects whatever from the injections.

EXPERIMENT 10.

In connection with his culture experiments Lourens states that the addition of an equal quantity of serum to bouillon furnishes a fluid which considerably increases the permeability of filters of the Berkefeld and Pasteur-Chamberland types. In order to test the correctness of this statement with regard to filters of the Pasteur-Chamberland type the following experiment was carried out:

A flask containing 250 c. c. of bouillon was inoculated with *B. choleræ suis* (H 2228 S) and incubated for forty-four hours. An equal amount (250 c. c.) of horse serum was then added to the bouillon culture and the mixture thoroughly shaken. Filtration was made through a new Pasteur-Chamberland F bougie. The filtrate was collected in seven portions of 70 c. c. each, the total amount of culture filtered being 490 c. c.

Table showing details of experiment 10.

Portion.	Amount.	Time of filtration.	Vacuum.	Result of incubation.
	c. c.	*Minutes.*	*Inches.*	
I	70	1	28	Filtrate free from *B. choleræ suis.*
II	70	1	28	Do.
III	70	1½	28	Do.
IV	70	3	28	Do.
V	70	7	28	Do.
VI	70	27	28	Do.
VII	70	60	28	Do.

After twenty-four hours in the incubator the filtrates all showed a slight opalescence, but there was no apparent clouding of the medium. After one week in the incubator the opalescence noted at the end of twenty-four hours had not increased and there was still no clouding of the filtrates.

Cultures were made from portions I, II, V, VI, and VII on various laboratory media, including Martin's serum-broth, with negative results.

Portions III and IV were removed from the incubator at the end of one week and subjected to a careful microscopic examination. Smear preparations stained with dilute carbol-fuchsin showed a finely granular material and occasional small coccus-like bodies like those noted in experiment 9. Cultures were made from each of these portions by adding 10 c. c. to flasks containing 400 c. c. each of beef broth. Rabbits were then given subcutaneous injections as follows: Rabbit 2428 (weight 2,160 grams) received 10 c. c. of portion III; rabbit 2429 (weight 2,935 grams) received 10 c. c. of portion IV.

After the removal by means of sterile pipettes of the portions used for the inoculation of cultures and rabbits, portions III and IV were then inoculated with *B. choleræ suis* (H 2228 S) and replaced in the incubator. Both portions showed well-marked clouding at the end of twenty-four hours, and microscopic examination revealed actively motile forms of *B. choleræ suis*, thus proving that the medium was not unfavorable to the growth of the bacillus.

The two flasks of bouillon which were inoculated with 10 c. c. each of portions III and IV failed to show any growth upon prolonged incubation, and the two rabbits inoculated with like amounts of the same portions showed no ill effects as a result of the inoculations.

The opalescence noted in the filtrates was evidently due to a slight precipitation of albuminous material from the blood serum.

This experiment would show that in the case of the Pasteur-Chamberland filter the addition of serum to the material filtered does not affect the permeability of the filter.

SUMMARY OF EXPERIMENTS WITH PASTEUR-CHAMBERLAND FILTERS.

The results of the experiments with Pasteur-Chamberland filters are summarized in the following table:

Summary of Pasteur-Chamberland filtrations.

No. of experiment.	Date begun.	Culture.		Grade of filter.	Amount of culture filtered.	Time occupied in filtration.	Vacuum.	Result of incubation.
		Number.	Age.					
					c. c.	*h. m.*	*Inches.*	
1	Feb. 24, 1904	Gp 3998 S.	24 hours ..	F	200	35	23	*B. choleræ suis* absent.
2	Mar. 2, 1904	.....do	.....do	F and B	200	45	21-22	Do.
3	June 6, 1905	Gp 4692 S.	7 days	B	100	37	20	Do.
4	Nov. 29, 1907	H 2199 S ..	2 days	F	[a]335	3 58	21	Do.
5	Nov. 30, 1907	.....do	18 hours...	F	300	11	20½	Do.
6	Dec. 3, 1907	.....do	24 hours...	F	525	1 23	20-25	Do.
7	Dec. 5, 1907	.....do	18 hours...	F	[a]600	2 4½	18-22½	Do.
8	Dec. 7, 1907	.....do	24 hours...	F	525	2 2½	8-23½	Do.
9	June 12, 1908	H 2450 S ..	26 hours...	F	350	39	20-23	Do.
10	Aug. 24, 1908	H 2228 S ..	44 hours...	F	490	1 40	28	Do.

[a] The portions which filtered through overnight under atmospheric pressure are not included in this table.

DISCUSSION OF PASTEUR-CHAMBERLAND FILTRATIONS.

In the experiments just described, nine Pasteur-Chamberland filters were tested with bouillon cultures of *B. choleræ suis* under an average vacuum of 20 to 25 inches. Five different strains of *B. choleræ suis* were used, and the cultures filtered varied in age from eighteen hours to seven days. The amount of culture filtered varied from 100 to 750 c. c., and in not a single instance could *B. choleræ suis* be detected in the filtrates.

In testing the filtrates for *B. choleræ suis* one or more, sometimes all, of the following methods were used: (1) Incubation of the filtrate, either entire or in fractions, for a considerable period of time; (2) the injection of large amounts of the filtrate into susceptible animals; (3) the introduction of collodion sacs containing filtrate into the abdominal cavities of susceptible animals; (4) microscopic examination, in both hanging-drop and stained preparations; (5) subcultures on various media favorable to the growth of *B. choleræ suis*. By none of these methods, however, could *B. choleræ suis* be detected in any of the filtrates, and the conclusion therefore seems justified that the organisms were completely restrained by the filters.

In view of the fact that all of the nine filters tested proved equally efficient in preventing the passage of *B. choleræ suis*, we may further conclude that the Pasteur-Chamberland filter may be depended on with safety to furnish as much as 500 to 600 c. c. of bacteria-free filtrates from bouillon cultures or correspondingly heavy suspensions of organisms approximating in size to *B. choleræ suis*. As it has been shown that bacteria are capable of growing through the walls of bacterial filters, it is best to limit the duration of the filtration to two or three hours. In two of the experiments recorded in this paper (Chamberland experiments 4 and 7) the apparatus was left in place and the portions which passed through the filters overnight were collected and incubated. These portions remained sterile, showing that in two instances at least *B. choleræ suis* did not grow through the walls of the Chamberland F filter in sixteen hours. Just how long it will require for different bacteria to grow through the Chamberland filter is a point which has not been fully investigated.

Although it does not seem probable in view of the perfect efficiency exhibited by the Pasteur-Chamberland filters in the experiments just described, it is nevertheless possible that an imperfect bougie may occasionally go out on the market, and for this reason it is important in all filtration experiments where bacteria-free filtrates are desired, even where the Pasteur-Chamberland filter is used, that all filtrates as well as filters be carefully tested.

Even if the experiments described in Bulletin 72 of this Bureau, previously mentioned, to which Lourens takes exception, had not

been sufficiently and carefully checked at the time they were made, the experiments with Pasteur-Chamberland filters described in the present paper would go far to prove that the filtrates employed in Bulletin 72 were absolutely free from *B. choleræ suis*, for in nearly all of the filtration experiments described in that bulletin the Pasteur-Chamberland type of filter was used almost exclusively, a new filter being used for each experiment, and the amount of filtrate collected never exceeded 500 cubic centimeters.

Lourens, however, criticises the filtration experiments described in Bulletin 72 on the ground that in testing the sterility of the filtrates cultures were made from the filtrates immediately after filtration. He claims that his experiments show that cultures made from filtrates immediately after filtration will almost always remain sterile, even though they be kept at incubator temperature for several weeks. This he explains on the ground that there may be so few organisms in the filtrate that they are missed in taking out portions for cultures. In making this criticism he apparently leaves out of consideration a point upon which he lays great emphasis in his conclusions, namely, the formation of granules sufficiently small to pass through the walls of a porcelain filter and capable of developing in the filtrates into *B. choleræ suis*. Now, if these granules be sufficiently small to pass through the walls of a porcelain filter, they should be present in the filtrates in considerable numbers and could hardly be missed in taking out portions for cultures. But aside from this apparent contradiction, we do not consider Lourens's criticism well founded, for as previously stated, repeated tests of the filtrates described in Bulletin 72 showed that cultures made with several times the amount of filtrate used for the inoculation of hogs did not show *B. choleræ suis* in a single instance, and guinea pigs and rabbits were unaffected by amounts sufficient to cause the death of hogs.

In the experiments described in the present paper the filtrates were held at incubator temperature for from one to two weeks before they were tested on animals, and supposing that only a very few organisms had passed through the filters into the filtrates these would certainly have had ample time to develop before the filtrates were tested. Where the entire filtrate was held at incubator temperature for a considerable time, as just explained, it did not seem necessary to make cultures from the filtrates in every instance, as it was repeatedly proven by subsequent inoculation that the filtrates themselves afforded a suitable medium for the growth of *B. choleræ suis* had this organism succeeded in passing through the walls of the filter.

Pasteur-Chamberland filters, which were subjected to a single cleansing according to the methods recommended by Lourens, were apparently unimpaired in efficiency, but it does not seem unlikely that repeated cleansings by these methods might in time affect the

pores of the filter and render it more permeable. Indeed, it is hard to explain the results of Lourens's Pasteur-Chamberland filtrations on any other ground unless it be that he used excessively high pressures in carrying out his filtrations. Lourens describes six experiments with bouillon cultures of *B. choleræ suis* in which Pasteur-Chamberland filters were used, and in four out of the six experiments *B. choleræ suis* appeared in the filtrates. In none of these six experiments does he appear to have used new filters, nor does he state how often his filters had been previously used and cleansed, but presumably they had been used a number of times. Lourens also fails to describe the manner in which he arranged his filters, whether for air pressure or vacuum, and nowhere in the course of his article does he record the amount of pressure or vacuum employed. It would appear, however, from the brief paragraph which he devotes to the technique of his filtration experiments that he made use of pressure in carrying out his filtrations, his filters being so arranged that air pressure could be applied to the fluid surrounding the filter and the fluid thus forced through the walls of the filter instead of being drawn through by means of suction. That he employed considerable pressure in carrying out his experiments is indicated from his statement that he experienced no difficulty in passing 500 c. c. of undiluted blood serum through a Berkefeld filter (size of filter not stated) in fifteen minutes or less, and that it required but little longer to pass the same amount of serum through a Chamberland F filter.

As this last statement in regard to the filtration of blood serum is so at variance with our own experience and the experience of other investigators who have found that blood serum filters with considerable difficulty, we have tested a number of Berkefeld and Chamberland filters with distilled water and blood serum, in order to determine their approximate rates of filtration.

RATE OF FILTRATION OF BERKEFELD AND PASTEUR-CHAMBERLAND FILTERS.

Employing a vacuum of 29 inches, it required about fifteen minutes to pass 500 c. c. of distilled water through a No. 7 Berkefeld laboratory cylinder, about ten minutes to pass the same amount through a Chamberland F filter, and from fifteen to twenty minutes to pass a like amount through a Chamberland B filter. With clear, limpid hog serum, which was first passed through a large No. 2 Berkefeld cylinder, in order to free it from all red blood cells, it required over three hours to pass 500 c. c. through a Berkefeld laboratory cylinder No. 7 under a vacuum of 29 inches and over four hours to pass 300 c. c. through a Chamberland F filter under the same vacuum. In the case of both filters the filtration of the blood serum proceeded with progressive slowness, as was shown by collecting the filtrates in separate portions of 100 c. c. each. In the case of the Berkefeld filter

the first 100 c. c. of serum passed the filter in six minutes, the second in nine minutes, the third in twelve, and the fourth in thirty minutes, whereas the fifth portion of 100 c. c. required two and one-half hours to pass the filter, at the end of which time the filter had virtually ceased to act. In the case of the Chamberland filter the first 100 c. c. of serum passed the filter in fifteen minutes, the second in one hour, while the third portion of 100 c. c. required three hours to pass the filter, at the end of which time the filter had become completely clogged and filtration had practically ceased.

In view of these results, it is difficult to see how Lourens succeeded in passing 500 c. c. of blood serum through a Chamberland F filter in fifteen minutes, unless he used very high pressure, and if he used such pressure it is conceivable that in his filtration experiments the clouding of his filtrates from *B. choleræ suis* was due to the fact that he actually forced the organisms themselves through the walls of his filters.

GRANULE FORMATION IN CULTURES OF B. CHOLERÆ SUIS.

With regard to the presence of granules in cultures of *B. choleræ suis*, Lourens states that only certain strains possess this property of breaking up into granules, but in those strains which are characterized by granule formation the peculiarity is a fixed one and persists after the organism has been cultivated on the different culture media and also after it has been passed through the animal body. He states that in his experiments only those cultures which were characterized by granule formation and polar staining passed through the filters, his theory being that the granules are sufficiently small to pass through the pores of the filters, and after passing through the filters these granules are capable of developing in the filtrates into the characteristic bacillary forms. He describes the granule formation as giving rise to coccus-like forms which may have the appearance of constricted bacilli or cocci lying in close juxtaposition, and now and then as three coccus-like bodies lying free or surrounded by a zone resembling a capsule.

Three of the five cultures used in the experiments described in the present paper were carefully examined for granules like those described by Lourens. The stock cultures of two of the strains experimented with were unfortunately lost in moving the laboratory and could not be examined for granules. In examining the cultures for granules, smear preparations were made from bouillon cultures and from the water of condensation of agar cultures. The films were carefully fixed by means of formalin and methyl alcohol without the aid of heat, so as to avoid any possible distortion from overheating, and were stained with dilute carbol-fuchsin, as recommended by Lourens. In the three cultures examined polar staining was observed

in all and granular forms were noted which corresponded in every particular with those described and pictured by Lourens. We must admit, therefore, the correctness of Lourens's observation as to the formation of granules in cultures of *B. choleræ suis*, and it is also possible that these granules may pass through the pores of an unglazed porcelain filter; but in view of the results of our Pasteur-Chamberland filtrations, where the filtrates were proven in every instance to be free from *B. choleræ suis*, although at least three of the cultures experimented with showed granule formation, we must conclude that the granules upon which Lourens lays so much stress are incapable of developing into the characteristic bacillary forms of *B. choleræ suis* and that they possess no significance in filtration experiments with this organism.

In our experiments with the Berkefeld filter, where *B. choleræ suis* developed in the filtrates, we believe that the organisms themselves passed through the walls of the filter.

SUMMARY OF RESULTS.

When bouillon cultures of *B. choleræ suis* were filtered through the smaller Berkefeld laboratory filters it was found that after a time—that is, after a certain amount of culture had been filtered—the organisms appeared in the filtrates. With a vacuum of 20 to 25 inches of mercury not more than 100 c. c. of bacteria-free filtrate could be obtained with these filters. When bouillon cultures of *B. choleræ suis* were filtered through Pasteur-Chamberland filters (F and B), the organisms did not appear in the filtrates in a single instance, although as much as 600 c. c. of culture was filtered in one instance. With a vacuum of 20 to 25 inches of mercury, the Pasteur-Chamberland filters (F and B) can be depended on to furnish from 500 to 600 c. c. of bacteria-free filtrate from bouillon cultures of *B. choleræ suis* when the time consumed in filtration does not occupy more than two hours.

Beef broth or bouillon is apparently unaltered by passage through a Berkefeld or Pasteur-Chamberland filter, and the absence of growth in filtrates from bouillon cultures of *B. choleræ suis* can not be explained on the supposition that filtration effects an alteration in the bouillon which renders it unfit for the growth of the organism. The addition of an equal volume of horse serum to a bouillon culture of *B. choleræ suis* did not facilitate the passage of the organisms through the Pasteur-Chamberland filter.

Rabbits were injected subcutaneously with 10 c. c. of filtered culture, and other rabbits were injected intravenously and intraperitoneally with 5 c. c. of filtered culture, but none of these animals showed any ill effects from the injections. Hogs weighing from 30 to 40 pounds were injected subcutaneously with 20 c. c. of filtered culture and intravenously with 10 c. c. of filtered culture, but were not

rendered sick thereby. Collodion sacs containing filtered culture were placed in the abdominal cavities of rabbits, but remained sterile.

Granules were noted in cultures of *B. choleræ suis* and in the filtrates from these cultures, but these granules did not develop in the filtrates nor in subcultures made from these filtrates. These granules were also shown to be incapable of development in the bodies of rabbits and hogs.

CONCLUSIONS.

In view of the results stated above, we must conclude—

1. That Pasteur-Chamberland filters F and B effectually prevent the passage of *B. choleræ suis*.

2. That the smaller Berkefeld laboratory cylinders vary in permeability.

3. That certain of the Berkefeld laboratory cylinders will prevent the passage of *B. choleræ suis* when a limited amount of material is filtered.

4. That the granules noted in cultures of *B. choleræ suis* have no significance in filtration experiments with this organism.

5. That in the filtration experiments described in Bulletin 72 the filtrates employed did not contain *B. choleræ suis*.

6. That hog cholera is due to an ultra-visible virus sufficiently small to pass through the pores of the Chamberland filter.

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